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Chemical and morphological analysis of explanted polyurethane vascular prostheses: the challenge of removing fixed adhering tissue

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During *in vivo* experiments to evaluate the biocompatibility and biostability of alternative biomaterials, the ideal protocol for the handling and preservation of the explanted material is often compromised in order to meet the needs of both the pathologist and the materials scientist. Explants surrounded by tissue are often fixed in formalin or glutaraldehyde to facilitate later pathological and histological analysis, but the subsequent removal of such fixed tissue from thermally sensitive and less chemically stable polymers, such as polyurethanes, poses major problems for the materials scientist, who does not wish to modify the chemical, physical or morphological characteristics of the underlying biomaterial. The present study has attempted to find a solution to this problem by exposing virgin specimens of the microporous polyurethane Vascugraft® vascular prosthesis to six different cleaning conditions, all known to be effective in removing fixed tissue. These conditions included the use of 20% aqueous potassium hydroxide solution for 48 h at room temperature, 5% sodium bicarbonate solution for 5 min at the boil, and 9, 10, 11 and 12 N hydrochloric acid for 48 h at room temperature. The appearance and chemical properties of the virgin and treated specimens were compared using electron spectroscopy for chemical analysis, Fourier transform infrared spectroscopy, gel permeation chromatography for molecular weight and differential scanning calorimetry techniques. The use of temperatures close to the boil resulted in the formation of a translucent, rubbery material with gross changes in the microporous and microfibrous structure. The strongly acidic and alkaline conditions caused a loss in the surface carbonate group content. In addition, 12 N hydrochloric acid reduced the molecular weight and urethane content. Consequently, 9 N hydrochloric acid is recommended as the cleaning agent of choice for removing fixed tissue from this type of microporous polyurethane. Control experiments on virgin material should also be included in any cleaning protocol. © 1996 Elsevier Science Limited

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The complete removal of encroaching tissue from explanted medical devices is necessary when studying the changes that may have occurred to the device as a result of implantation. Traditional fixation methods using formaldehyde or glutaraldehyde cause the tissue to become tightly attached to the structure of the prosthesis. The difficulty of completely removing all such adhering cross-linked tissue without modifying the surface and bulk properties of the underlying biomaterial imposes severe limitations on the reliability of studies attempting to measure *in vivo* changes in the chemical, physical and structural properties of implantable devices. We have found this difficulty particularly challenging when studying microfibrous vascular prostheses made from polyurethanes. This is because their delicate microfibrous structure is easily damaged, they maintain a strong attachment to adhering tissue and polyurethanes themselves are susceptible to chemical degradation by the cleaning agents.

Over the years, various methods of cleaning explanted samples have been developed in our laboratory[1–4]. Sodium bicarbonate solutions have been

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used since before 1980 to clean explanted polyethylene terephthalate (PET) vascular grafts[1], and the cleaned specimens are suitable for analysis by various techniques such as scanning electron microscopy (SEM), differential scanning calorimetry (DSC), bursting strength testing and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Other detergents and cleaning procedures have been attempted on explanted polyurethane materials and have been found to be more or less effective in removing most of the tissue[2]. However, the disadvantage of these cleaning methods is that they are either too harsh for certain polymers such as polyurethane, or they do not completely remove all the adhering tissue, thereby affecting the results of some of the more sensitive surface analytical methods, such as electron spectroscopy for chemical analysis (ESCA)[2,3]. Recently, a new enzymatic cleaning method has been shown to be useful in preparing very clean explanted specimens that are suitable for analysis by most conventional surface analytical methods, including ESCA[4]. However, this method is only effective with samples that are not cross-linked. The objective of this study, therefore, was to develop a method that would be suitable for cleaning and removing all fixed tissue from explanted prostheses made from less stable polymers such as polyurethanes without causing structural or chemical modifications to the polymer.

In this study, virgin polyurethane vascular prostheses were subjected to different chemical cleaning regimes before carrying out microscopic and chemical analyses. By monitoring any chemical and structural modifications in the polyurethane that were induced by the cleaning chemicals, it was anticipated that the conclusions would recommend which cleaning protocol is the most innocuous for this type of biomaterial.

MATERIALS AND METHODS

Material

Polyurethane vascular grafts (Vascugraft™, Braun-Melsungen AG, Melsungen, Germany) were used as supplied in sterile packages. Details of the structural, physical and chemical characteristics of the Vascugraft prosthesis have been presented elsewhere[5–7]. Briefly, this particular polyurethane prosthesis has a non-woven microfibrous structure, and chemically is composed of non-aromatic hard segments and carbonate soft segments.

Chemical cleaning treatments

The following six cleaning treatments are known to be effective in removing cross-linked biological tissue from synthetic explanted biomaterials[8].

Sodium bicarbonate
A coupon of the virgin polyurethane prosthesis was boiled with continuous stirring in an aqueous solution of sodium bicarbonate (5%) for 15 min. After leaving the solution to cool to room temperature, the sample was removed and rinsed thoroughly in double deionized water. It was then dried in a vacuum oven at room temperature until a constant weight was achieved.

Potassium hydroxide
A virgin polyurethane sample was placed in a beaker containing an aqueous solution of potassium hydroxide (20%) at room temperature. The contents of the beaker were stirred continuously for 48 h. The specimen was rinsed in double deionized water and dried in a vacuum oven at room temperature until a constant weight was achieved.

Hydrochloric acid
Other virgin polyurethane samples were placed in beakers containing hydrochloric acid at concentrations of 9, 10, 11 and 12 N and stirred continuously for 48 h at room temperature. After rinsing in double deionized water, the specimens were vacuum dried at room temperature until a constant weight was achieved.

Analysis of chemically treated prostheses

Visual observations
Before undertaking microscopic and chemical analyses, the cleaned samples were inspected visually and their appearance was compared with an untreated virgin sample.

Microscopic observations through SEM
Virgin and chemically cleaned specimens were sputter coated with gold–palladium and then observed by means of a Jeol JSM 35CF scanning electron microscope (Soquelec Ltd., Montréal, Québec, Canada) operated at 15 eV accelerating voltage. Both internal and external surfaces of the prostheses were examined at different magnifications.

Surface chemical analysis by ESCA
A VG ESCALAB MKII X-ray photoelectron spectroscope (VG Scientific, East Grinstead, West Sussex, UK) was used to analyse the surface chemistry of the specimens before and after the different cleaning treatments. Carbon 1s spectra were recorded using a Mg K_α radiation source operating at 20 kV and 15 mA. Both survey scans and high resolution scans were recorded using pass energies in the analyser of 50 and 20 eV, respectively. A vacuum of about 10^{-9} kPa was maintained in the sample chamber during each scan. The C_{1s} spectrum was recorded at both the beginning and the end of the measurement for each specimen. Comparing the two spectra confirmed that there was no specimen surface damage caused by the X-rays. The internal surface of each specimen was analysed to a sampling depth of about 5 nm[9].

Surface chemical analysis by ATR-FTIR
The infrared absorption spectra of the internal surfaces of the virgin and chemically cleaned prostheses were obtained with a Bomem DA3-02 Fourier-transform infrared spectrophotometer (Bomem Inc., Québec, Canada) in conjunction with a KRS-5 crystal and a Wilks attenuated total reflectance, ATR, attachment. The angle between the incident light and the sample

surface was held constant at 45°. The effective sampling depth was about 0.5–3.0 μm for the scanning range from 4000 to 700 cm^{-1} [10], assuming a refractive index for polyurethane of 1.5[11]. Assignment of the absorption peaks of the Vascugraft polyurethane material was made according to previously published data[6].

Analysis of bulk morphology by DSC

A Perkin–Elmer Model 7 differential scanning calorimeter (Perkin–Elmer Corporation, Montréal, Québec, Canada) was used to measure any changes in thermal properties between the virgin and chemically cleaned prostheses. Samples weighing between 10 and 20 mg were analysed at a scanning rate of 10°C min^{-1}. The temperature scanning range was from −100 to 200°C using liquid nitrogen as the cooling medium and helium as the inert atmosphere surrounding the sample. The DSC system was calibrated against a known mass of indium.

Molecular weight analysis by gel permeation chromatography

The number-average and weight-average molecular weights of the specimens exposed to 9 and 12 N HCl for 48 h were measured by means of a Waters Millipore® model 590 gel permeation chromatograph (Waters Associates, Milford, MA, USA). An Ultrastyragel™ column with a pore size of 10 nm (Millipore Canada Ltd., Waters, Ville St-Laurent, Québec, Canada) and a differential refractometer were connected to the chromatograph for separation and detection. Filtered tetrahydrofuran (THF) was used as the mobile phase and flow rate of 1.0 ml min^{-1} was maintained throughout the experiment. Each polyurethane specimen was dissolved in filtered THF and made up to a 1% solution. Eighty microlitres of this solution were filtered before being injected into the flow system. Three injections were made for each specimen.

RESULTS

Macroscopic observations

After 15 min in the boiling sodium bicarbonate solution, the Vascugraft specimen changed from having a white opaque appearance and paper-like feel to a semi-transparent, rubber-like material. Shrinkage occurred in both radial and axial directions, while the wall thickness became noticeably greater.

The treatments with potassium hydroxide and hydrochloric acid did not produce any visible changes to the prosthesis.

SEM observations

The SEM image of the internal surface of the untreated virgin prosthesis at ×200 magnification is shown in *Figure 1*. Microscopic observations of both internal and external surfaces of the potassium hydroxide and hydrochloric acid treated specimens revealed a normal microfibrous and microporous surface structure free from apparent damage. Representative views of their respective internal surfaces are shown in *Figures 2* and *3*. Following treatment in sodium bicarbonate solution at the boil the specimen experienced gross changes in morphology, with a loss of the microfibrous and microporous structure and the formation of holes measuring about 100 μm across (*Figure 4*).

ESCA observations

Table 1 summarizes the ESCA results for the virgin and the chemically cleaned specimens. The carbonate content was found to decrease after either alkaline or acid exposure. A slight decrease in oxygen was also detected when alkaline or strongly acidic conditions were used. At the same time, the surface urethane content increased along with a corresponding increase in nitrogen. These findings suggest that hydrolysis of

Figure 1 SEM photomicrograph of the internal surface of a virgin (as-received) Vascugraft® prosthesis.

Table 1 Surface composition of virgin and chemically cleaned Vascugraft® prostheses measured by ESCA

Chemicals	Elemental composition (%)					C_{1s} composition (%)			
	C	O	N	Si	Others	CC	CO	NCOO	OCOO
Virgin*	81.0	17.8	1.2	—	—	76.2	15.8	1.1	6.9
5% $NaHCO_3$	79.7	16.3	1.5	1.6	0.9	79.1	15.5	1.3	4.1
20% KOH	80.3	17.0	2.7	0.2	—	77.4	16.2	2.1	4.3
Virgin*	76.7	21.2	1.4	0.8	—	70.2	20.8	1.5	7.5
9 N HCl	76.6	21.5	1.8	—	0.1	68.7	22.2	2.3	6.7
10 N HCl	75.7	21.7	2.1	0.5	0.1	68.2	22.9	2.6	6.3
11 N HCl	76.2	21.6	1.8	0.1	0.2	67.9	22.8	2.4	6.9
12 N HCl	77.7	20.3	1.7	0.3	0.2	70.8	19.9	3.1	6.2

*The results of the virgin controls are different because the samples were obtained from two different batches.

surface carbonate groups occurred, combined with movement of the hard segments towards the surface. No significant change in other functional groups was detected. Although the alterations resulting from the milder acid treatments were marginal, the strongest acid concentration (12 N) was found to produce a significantly lower carbonate and higher urethane content. It was also found that the decrease in carbonate content was significantly greater in alkaline (41%) compared with acidic (17%) conditions.

Figure 2 SEM photomicrograph of the internal surface of the Vascugraft® prosthesis after exposure to 20% potassium hydroxide solution for 48 h at room temperature.

Figure 3 SEM photomicrograph of the internal surface of the Vascugraft® prosthesis after exposure to 12 N hydrochloric acid for 48 h at room temperature.

Figure 4 SEM photomicrograph of the internal surface of the Vascugraft® prosthesis after exposure to 5% sodium bicarbonate solution for 15 min at the boil.

ATR-FTIR observations

The relative intensities of the carbonyl absorption peaks in the carbonate and urethane structures are illustrated in *Figures 5* and *6*. The following three peaks were selected to monitor the chemical changes at the sample surface: non-hydrogen-bonded carbonate (1738 cm^{-1}), hydrogen-bonded carbonate (1717 cm^{-1}) and hydrogen-bonded urethane (1690 cm^{-1}). Because the methylene group concentration was expected to remain stable during the different cleaning treatments, the absorption at 1462 cm^{-1} was used as the thickness band for reference purposes.

Figures 5 and *6* show that exposure to potassium hydroxide had no effect on these absorption peaks. However, 12 N hydrochloric acid did, reducing the intensity of the urethane and hydrogen-bonded carbonate absorptions, while increasing the non-hydrogen-bonded carbonate absorption. This suggests that under strong acidic conditions, some of the urethane groups are hydrolysed and some of the hydrogen-bonded carbonate groups are converted to non-hydrogen-bonded groups. Exposure to sodium

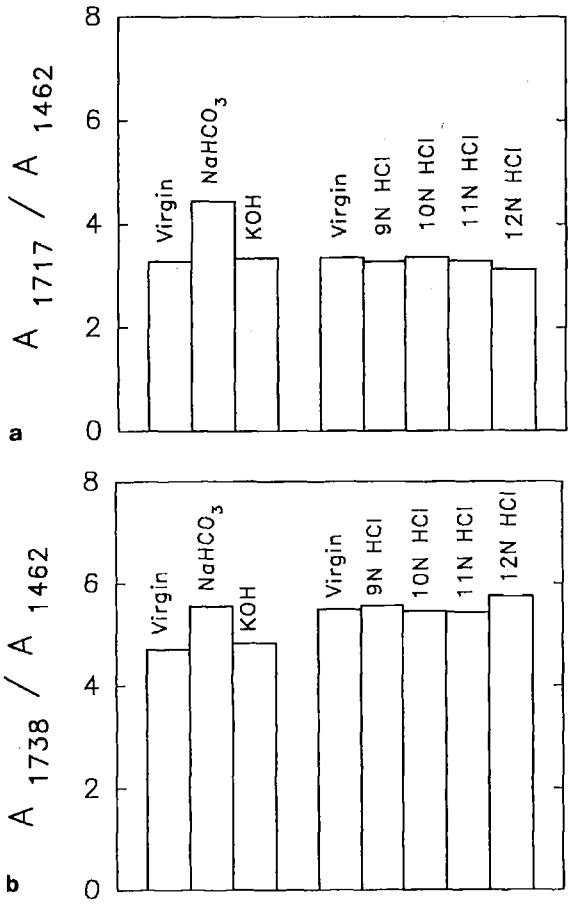

Figure 5 The effect of various cleaning treatments on the carbonate carbonyl absorption of the Vascugraft® prosthesis expressed as ratio of absorptions measured by ATR-FTIR. **a**, Hydrogen-bonded carbonyl group; **b**, non-hydrogen-bonded carbonyl group.

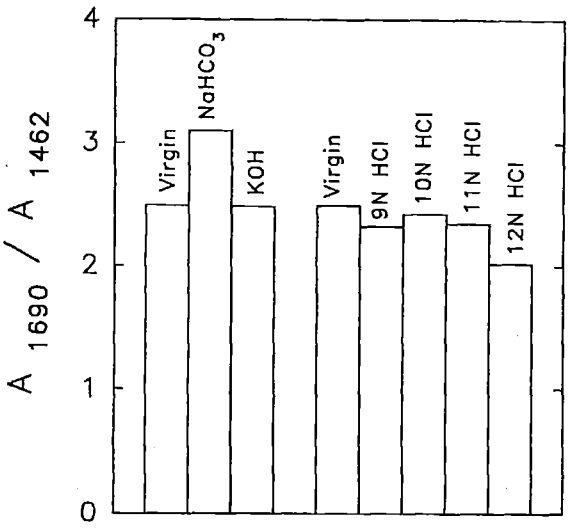

Figure 6 The effect of various cleaning treatments on the hydrogen-bonded urethane carbonyl group of the Vascugraft® prosthesis expressed as a ratio of absorptions measured by ATR-FTIR.

bicarbonate at the boil appeared to increase all three absorption intensities. A simpler explanation is perhaps a decrease in the methylene group concentration (1462 cm^{-1}) at the surface owing to a reorganization of the macromolecular chains during the boiling process.

DSC observations

As shown in *Table 2*, there was a tendency after both alkali and acid exposure for endotherms T_2 and T_3 to shift to a higher, and endotherm T_1 to shift to a lower, temperature. However, these shifts were not significant except for the specimen exposed to 12 N hydrochloric acid.

Gel permeation chromatography observations

Of the four acid conditions used in this experiment, both the lowest and the highest HCl concentrations significantly decreased the weight-average molecular weight of the Vascugraft material (*Table 3*). In fact, the number-average molecular weight and the polydispersity were also decreased for the specimen exposed to 12 N HCl.

Table 2 Thermal behaviour of the virgin and chemically cleaned Vascugraft® prostheses measured by DSC (°C)

Chemicals	T_g	T_1	T_2	T_3
Virgin*	−54.9	37.2	57.0	87.9
20% KOH	−54.8	36.0	59.3	88.6
Virgin*	−66.5	22.8	39.7	73.2
9 N HCl	−66.4	19.7	41.8	75.5
10 N HCl	−66.8	19.5	42.1	76.3
11 N HCl	−67.2	21.2	42.2	73.7
12 N HCl	−66.7	18.5	51.7	90.7

*Virgin controls for the KOH- and HCl-treated specimens were from different batches.

DISCUSSION

In order to study the surface chemistry of explanted prostheses, it is necessary to remove all the tissue that may have grown over and within the prosthetic structure. In the event that the explant has been treated with a fixative agent after retrieval, such as formaldehyde or glutaraldehyde, the tissue will be cross-linked and the only effective way of completely removing it is to use hydrolytic chemicals. Depending on the degree of cross-linking, strong chemicals and/or extreme hydrolysis conditions may be required. Under these circumstances, caution should be exercised when using such severe chemical conditions because of the risk of modifying the inherent chemical and physical structure of the biomaterial. Inevitably, carefully controlled experiments are required with the inclusion of the cleaning of a virgin reference specimen in order to distinguish between those alterations to the prosthesis material owing to implantation from those caused by the cleaning treatment.

The effects of six different cleaning conditions have been investigated on the chemical, physical and structural properties of the Vascugraft polyurethane vascular prosthesis. The greatest morphological change was observed following the sodium bicarbonate treatment at the boil. While this method has been used successfully in our laboratory to clean fixed tissue from explanted PET vascular prostheses without any significant changes to the chemistry of the prosthesis, the high temperature causes the polyurethane specimen to shrink and completely change its microfibrous structure. This is due to the greater thermal stability of PET compared with polyurethanes. PET monofilaments are highly crystalline and have a glass transition temperature in the 100–130°C range and a melting point of about 260°C[12]. Exposure to temperatures around 100°C is therefore not harmful. On the other hand, elastomeric polyurethanes are more heat sensitive and, for example, the polyurethane used to construct the Vascugraft prosthesis has several major endotherms below 100°C (*Table 2*). Above these transition temperatures the hard segment domains no longer function as physical cross-links, which explains why the Vascugraft prosthesis softens and shrinks when boiled in sodium bicarbonate solution. The other five cleaning conditions did not cause any apparent effects on the microfibrous structure of the prosthesis.

The chemical stability of elastomeric polyurethanes does not compare favourably with that of fibre forming PET. The ester group in the PET polymer is protected by its high crystallinity and its hydrophobic aromatic

Table 3 Molecular weight results of virgin and hydrochloric acid-treated Vascugraft® prostheses measured by gel permeation chromatography

HCl condition	M_n	M_w	M_w/M_n
Virgin control	52 300 ± 2500	138 000 ± 300*	2.65 ± 0.13
9 N HCl	42 400 ± 4100	104 500 ± 3500*	2.48 ± 0.32
12 N HCl	41 400 ± 4100	92 400 ± 5300*	2.24 ± 0.09

*Significant difference between HCl-treated and virgin specimens at a confidence level of 99% (two-tailed student *t*-test, d.f. = 4).

structure, which prevents the absorption and ingress of water and acidic or alkaline components into the material. While the thermal analysis of the Vascugraft prosthesis pointed to some ordered hard segment domains, no significant quality of a crystalline hard segment was found[6]. In a previous study, we have observed that the Vascugraft prosthesis absorbs moisture during incubation in an aqueous environment[13], implying that small molecules and ions such as water, OH^- and H^+ will not only be absorbed at the surface but will also penetrate inside the prosthetic material. The decrease of carbonate content on the sample surface after incubation in either alkaline or acidic environments was determined with ESCA in this experiment. It was most likely that this decrease was induced by hydrolysis of the carbonate groups catalysed by the OH^- or H^+ species. The loss in molecular weight caused by the exposure to HCl strongly supports this conclusion. The measured degradation of polyurethane molecules might also indicate that the hydrolysis was not only a surface but also a bulk phenomenon.

The surface of the Vascugraft prosthesis was unaffected by alkali and acid exposure provided the temperature or the acid concentration were not too high. This was confirmed by both ATR-FTIR and DSC results, which showed little difference between the cleaned specimens and their virgin controls when low acid concentrations and ambient temperatures were used. There was no apparent damage to the polyurethane microfibres caused by these chemical incubations. Moreover, the acidic environments had much less effect on the surface carbonate groups than the alkaline one. Therefore, certain cleaning methods are more suitable for removing fixed tissue from polyurethane explants than others. In the light of the findings from this study, 9 N hydrochloric acid is to be recommended, since it is the least harmful of the six cleaning conditions investigated.

CONCLUSIONS

The chemicals used to clean fixed tissue from explanted vascular prostheses can affect the inherent physical, chemical and morphological properties of the prosthetic material, especially when they are made from thermally sensitive and less chemically stable polyurethanes. The use of temperatures near the boil results in major changes to the microfibrous and microporous morphology. Strong acidic and alkaline conditions reduce the carbonate group content near the surface of the prosthesis. In addition, strong acid treatments also reduce the molecular weight and urethane content of the material. The results from this study recommend the use of 9 N hydrochloric acid as the most suitable cleaning agent for removing fixed tissue from explanted Vascugraft polyurethane prostheses. It is also recommended that control 'cleaning' experiments are carried out on virgin material in order to distinguish between the effects of implantation from those of the cleaning agent.

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